ORIGINAL ARTICLE

Effect of dietary α-tocopherol + ascorbic acid, selenium, and iron on oxidative stress in sub-yearling Chinook salmon (*Oncorhynchus tshawytscha* Walbaum)

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Summary

A three-variable central composite design coupled with surface-response analysis was used to examine the effects of dietary α-tocopherol + ascorbic acid (TOCAA), selenium (Se), and iron (Fe) on indices of oxidative stress in juvenile spring Chinook salmon. Each dietary factor was tested at five levels for a total of fifteen dietary combinations (diets). Oxidative damage in liver and kidney (lipid peroxidation, protein carbonyls) and erythrocytes (erythrocyte resistance to peroxidative lysis, ERPL) was determined after feeding experimental diets for 16 (early December) and 28 (early March) weeks. Only TOCAA influenced oxidative stress in this study, with most measures of oxidative damage decreasing (liver lipid peroxidation in December and March; ERPL in December; liver protein carbonyl in March) with increasing levels of TOCAA. We also observed a TOCAA-stimulated increase in susceptibility of erythrocytes to peroxidative lysis in March at the highest levels of TOCAA. The data suggest that under most circumstances a progressive decrease in oxidative stress occurs as dietary TOCAA increases, but higher TOCAA concentrations can stimulate oxidative damage in some situations. Higher levels of TOCAA in the diet were required in March than in December to achieve comparable levels of protection against oxidative damage, which may have been due to physiological changes associated with the parr-smolt transformation. Erythrocytes appeared to be more sensitive to variation in dietary levels of TOCAA than liver and kidney tissues. Using the March ERPL assay results as a baseline, a TOCAA level of approximately 350–600 mg/kg diet would provide adequate protection against lipid peroxidation under most circumstances in juvenile Chinook salmon.

Introduction

Reactive oxygen species (ROS; e.g. hydrogen peroxide and superoxide anion) can be highly reactive and have been implicated in the pathology of many diseases (McCall and Frei, 1999). Organisms possess enzyme systems and small-molecular-weight molecules with antioxidant capabilities that are capable of neutralizing ROS and protect against their adverse effects (Machlin and Bendich, 1987). However, ROS generation can exceed the neutralization capacity of antioxidant defences, and oxidative stress can occur (Sies, 1985). Oxidative stress has been identified as a causative agent in a number of pathologies and diseases in fish, such as cataracts (Waagbø et al., 2003), parasitic infection (Kwon et al., 2002; Dautremepuits

et al., 2003), swim bladder inflammation (Nemcsók et al., 1993), muscular dystrophy (Poston et al., 1976), liver lipoid degeneration (Roald et al., 1981), and stress-mediated peroxidative damage to tissues (Welker and Congleton, 2003, 2004). Salmonids may be particularly prone to oxidative stress due to abundant tissue concentrations of highly-peroxidizable, unsaturated n-3 fatty acids (HUFA; Henderson and Tocher, 1987), which are highly susceptible to damage by oxygen radicals (Lygren et al., 1999).

Nutrition plays a key role in maintaining the pro-oxidant–antioxidant balance of fish, and several vitamins and minerals are important in the antioxidant defences of aquatic organisms (Cowey, 1986). Vitamin E (tocopherol) is the major membrane-bound lipid-soluble antioxidant (Machlin and Bendich, 1987), while vitamin C (ascorbic acid) is an important water-soluble antioxidant (Levine, 1986) in animals. These two antioxidant compounds interact synergistically to protect water and lipid phases against oxidation in organisms (Hamre et al., 1997). In animals, megadoses of ascorbic acid (Barja et al., 1996) and vitamin E (Mukay et al., 1993; Kaewsrithong et al., 2001) can be pro-oxidant. Selenium is an essential trace mineral obtained partly from the surrounding water (Lall and Bishop, 1977) but mostly from the diet (Halver, 2002). The importance of selenium to oxidative stress is its requirement at the active sites of the antioxidant enzymes glutathione peroxidase and phospholipid hydroperoxide glutathione peroxidase (Felton et al., 1996), which catalyze the reduction of hydroperoxides and peroxides to less reactive alcohols and water (Felton et al., 1996). There is also a synergistic action between vitamin E and selenium in salmonids (Bell and Cowey, 1985).

Iron, although a physiologically important transition metal required by the antioxidant enzyme catalase (Rice-Evans et al., 1991), can catalyze the formation of ROS when in dietary excess (Kehrer, 1993). High levels of iron in fish feeds can adversely affect the immune system, reduce growth rates, and catalyze the oxidation of dietary lipids, and historically, iron levels in commercial salmon feeds have ranged from 200 to 1000 mg/kg diet (Desjardins et al., 1987). More recently, Maage (1994) reported iron levels in Atlantic salmon (*Salmo salar* L.) diets were 51–515 mg/kg diet. These levels generally exceed the recommended minimum dietary requirements of 60–100 mg/kg diet for Atlantic salmon (Andersen et al., 1996) and 60 mg/kg diet for rainbow trout (National Research Council (NRC), 1993). In addition, many commercial salmonid diets are

12–16% lipid, of which 20% or more is polyunsaturated fatty acid (PUFA; Desjardins et al., 1987). In recent years, the lipid content of some commercial salmonid diets has increased from 17 to 35% (Torstensen et al., 2001). Elevated dietary iron without adequate levels of antioxidant in feed may produce high levels of toxic lipid peroxides in fish feed, which upon feeding may initiate peroxidation of tissue lipids (Dix and Aikens, 1993). The importance of nutrition in the pro-oxidant–antioxidant balance may be highly critical in intensive aquaculture, where fish are almost completely reliant upon vitamins and minerals present in diets and are often exposed to conditions favourable for oxidative stress, such as poor water quality, disease and stress.

Research on the interrelationships of three or more dietary nutrients is often not feasible using standard factorial statistical designs, in which the number of experimental units increases exponentially with the addition of each dietary factor to be tested. In many situations, however, a fractional replicate of a factorial design, which can reduce the dimensionality of the problem by looking at the marginal response surface independently of certain effects, can be substituted (Mead and Pike, 1975). We employed a three-variable central composite design coupled with surface-response analysis in this study to determine the effects of dietary α-tocopherol + ascorbic acid (TOCAA), selenium (Se) and iron (Fe) on indices of oxidative stress in spring Chinook salmon (*Oncorhynchus tshawytscha* Walbaum). We also examined possible interactions between these dietary nutrients. We hypothesized that dietary TOCAA and Se would decrease and Fe would increase oxidative stress in juvenile Chinook salmon. In addition, we expected a synergistic reduction in oxidative stress with increasing dietary concentrations of TOCAA and Se, but a synergistic increase in oxidative damage with increasing levels of TOCAA and Fe.

Materials and methods

Fish rearing

Spring Chinook salmon parr ($n = 3{,}000$; 1.5 ± 0.3 g mean wet weight; Willamette River stock) were obtained from the Smith Farm Aquaculture Facility at Oregon State University, Corvallis, OR, USA in early February. Parr were reared in two 1000-l tanks and fed Biodiet Starter diet for salmon and trout (Bio-Oregon, Warrenton, OR, USA; 1.3 mm) for approximately 6 weeks and Biodiet Grower diet (Bio-Oregon; 2.0 mm) thereafter for 12 weeks until

the beginning of the study. Four weeks prior to the start of the study, approximately 50 parr were randomly distributed to each of 32 tanks (200-l) and allowed to acclimate on the basal diet. After the acclimation period, experimental diets were randomly assigned to tanks and the study was initiated. The initial mean weight and fork length of the fish were approximately 15.1 ± 1.2 g and 106 ± 2 mm. Fish were fed twice daily (09:00 and 17:00 hours) for 28 weeks at a rate based on the caloric content of dry feed (3150 kcal/kg digestible energy) and calculated as described by Klontz et al. (1980). Fish were weighed once per month and feed allowances adjusted accordingly. Beginning in August, fish were fed 1.6 mm feed pellets for 7 weeks and 3.2 mm pellets thereafter until the completion of the study. Aeration was supplied by compressed air through airstones, and water inflow was 4.0 l/min. Water temperatures were controlled by mixing water from chilled and unchilled lines and ranged from 8 to 11 °C. The water was obtained from deep wells and had a total hardness of 98 mg/l (58 mg/l as calcium) and a pH of 7.8.

Experimental diets

Semi-purified test diets based on the Pacific salmon modified H-440 formulation (National Research Council (NRC), 1993) were manufactured at the Hagerman Fish Culture Station, University of Idaho, Hagerman, ID, USA (Table 1). Only the levels of α-tocopherol, ascorbic acid, Se and Fe varied among the individual test diets; concentrations of all other ingredients did not vary. No artificial antioxidants are added to dietary menhaden oil after refinement (natural α-tocopherol = 4 mg/kg oil). All other dietary ingredients were obtained form Sigma Chemical Corporation (St Louis, MO, USA) unless otherwise stated. Fifteen test diets were used during the study. Individual diets and the corresponding levels of α-tocopherol, ascorbate, Se, and Fe are given in Table 2. The level of TOCAA indicated in Table 2 is the level of α-tocopherol and ascorbic acid supplemented in the diet (e.g. in diet 1, 608.1 mg/kg of α-tocopherol and 608.1 mg/kg ascorbic acid were added to the diet).

Fish and tissue sampling

Fish were sampled after 16 weeks (early December) and 28 weeks (early March) of feeding experimental diets. Fish sampled at the end of the experiment in March exhibited body silvering and loss of parr

Table 1 Pacific salmon modified H-440 diet

Ingredient	Percentage of diet
Casein (vitamin-free)*	40.8
Gelatin*	8.0
Dextrin*	19.5
α-Cellulose†	4.7
Super-refined menhaden oil‡	15.0
Choline chloride (60%)	0.5
Amino acid mixture§	4.4
Vitamin premix¶	3.1
Mineral premix**	4.0
Tested nutrients††	

*United States Biochemical, Cleveland, OH, USA.
†Sigma Chemical Corp., St. Louis, MO, USA.
‡Croda, Fullerton, CA, USA.
§Amino acid supplied as mg/kg diet: l-arginine-HCl, 10.0; DL-lysine, 10.0; DL-methionine, 4.0; l-phenylalanine, 5.0; l-threonine, 10.0.
¶Vitamin supplied per as mg/kg diet: retinol palmitate, 5.0; cholecalciferol, 0.025; menadione
sodium bisulphite, 10.0; thiamin, 30.0; riboflavin, 25.0; pyridoxine mono-HCl, 30.0; cyanocobalamin, 0.02; nicotinic acid, 200.0; d-pantothenic acid, 50.0; d-biotin, 1.0; folic acid, 10.0; myo-inositol, 500.0.
**Supplied as g/kg diet: KCl, 15.0; CaHPO$_4$ dibasic, 12.0; MgSO$_4$, 2.58; NaCl, 3.0; MnSO$_4$•H$_2$O, 0.20; CuSO$_4$•5H$_2$O, 0.005; ZnSO$_4$•H$_2$O, 0.040; KI, 0.010.
††Supplied as: α-tocopherol acetate; ascorbate poly-phosphate; Na$_2$SeO$_3$; FeSO$_4$•7H$_2$O. The levels of tested nutrients in each individual diet are in Table 2.

marks and appeared to be undergoing smoltification. Three fish were sampled randomly from each tank and placed in a lethal dose of buffered MS-222 (200 mg/l tricaine methanesulfonate; Argent Chemical Laboratories, Redmond, WA, USA). External anomalies, wet weight (nearest 0.1 g), and fork length (nearest 1 mm) were recorded for each fish. Whole blood was taken from the caudal vasculature with a 1-ml heparinized (air-dried; 800 U/ml) syringe fitted with a 20-gauge needle. Blood samples were immediately placed on ice and subsequently centrifuged at 2000 g to separate plasma and blood cells. After removal of the plasma and white blood cells coat, the remaining blood cells (primarily erythrocytes) were placed on ice and analysed for resistance to peroxidative lysis. After bleeding, the liver and kidney were removed and sectioned for use in bioassays. Each tissue section was placed into appropriately labelled tubes and frozen on dry ice. Tissue samples were stored at −80 °C for use in oxidative stress bioassays.

Analyses

The assay for protein carbonyl (PC) content of tissues, an indicator of peroxidative damage to

Table 2 Levels of tested nutrients supplemented in experimental diets

Diet	Dietary Level (mg/kg)*		
	TOCAA	Se	Fe
Three replicate tanks/diet (factorial portion of design)			
1	608.1	7.20	606.1
2	608.1	1.90	183.9
3	191.9	7.20	183.9
4	191.9	1.90	606.1
5	608.1	7.20	183.9
6	608.1	1.90	606.1
7	191.9	7.20	606.1
8	191.9	1.90	183.9
Two replicate tanks/diet (centre point)			
9	400.0	4.55	395.0
One tank/diet (axial portion of design)			
10	50.0	4.55	395.0
11	750.0	4.55	395.0
12	400.0	0.10	395.0
13	400.0	9.00	395.0
14	400.0	4.55	40.0
15	400.0	4.55	750.0

See text for explanation.

*TOCAA, α-tocopherol + ascorbic acid (the level of TOCAA indicated is the level of α-tocopherol and ascorbic acid supplemented in the diet (e.g. in diet 1, 608.1 mg/kg of α-tocopherol and 608.1 mg/kg ascorbic acid were added to the diet); Se, selenium; Fe, iron.

proteins, was performed according to Reznick and Packer (1994). Briefly, aliquots of extracted proteins from a tissue sample were added to 2.5 M HCl (control blank) and 10 mM dinitrophenylhydrazine (DNPH) in 2.5 M HCl and incubated for 1 h. Next, proteins were precipitated with 50% trichloroacetic acid (TCA) and washed three times with ethanol:ethylacetate (1:1; wt:vol) and then three times with 10% TCA. The final precipitate was dissolved in 6 M guanidine hydrochloride, and the spectrum of the DNPH-treated sample versus the HCl control was followed with a Shimadzu UV-1201 variable wavelength spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD, USA) at an absorbance of 355–390 nm. The concentration of carbonyl groups was calculated by multiplying the peak absorbance by 45.45 nmol/ml (derived from the extinction coefficient $\epsilon = 22\,000/\text{M}$ of DNPH). Total protein in extracts was determined with a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) and bovine serum albumin (BSA) standard curve and used to express tissue carbonyl content per mg protein. The carbonyl assay was not performed for liver samples collected at 16 weeks (December); samples intended for carbonyl analysis were used

instead to repeat the 16-week lipid peroxidation analyses, due to initial problems with this assay.

Total lipids were extracted according to Erickson (1993) by homogenizing frozen, wet tissue samples for 30 s in 20 parts (wt/vol) ice cold dichloromethane:methanol (2:1; vol/vol) and 0.01% butylated hydroxytoluene (BHT; to prevent *in vitro* peroxidation) with a tissue homogenizer (Ultra-Turrax T8, IKA Works, Wilmington, NC, USA). The ferrous oxidation-xylenol orange (FOX) method was used to quantify lipid hydroperoxides (Shantha and Decker, 1994; Burat and Bozkurt, 1996; E. A. Decker, Department of Food Science, Chenoweth Lab, University of Massachusetts, Amherst, MA, USA, personal communication) with slight modifications. Dichloromethane:methanol (2:1; vol/vol) rather than water was used as the assay solvent system, and lipid peroxidation was expressed as hydrogen peroxide equivalents (per g of tissue total lipid) by replacing the Fe^{3+} calibration curve with one utilizing hydrogen peroxide. After incubation of extracts with xylenol orange and Fe^{3+} for 5 min, 200 μl of the reaction mixture was pipetted into the wells of a 96-well flat-bottomed microplate, and absorbance at 562 nm was read (Kinetic Microplate Reader EL312E, Bio-Tek Instruments, Winooski, VT, USA). The lipid content of extracts was determined by the sulfo-phospho-vanillin reaction (Frings et al., 1972) by estimation from a menhaden fish-oil (Sigma, St Louis, MO, USA) calibration curve.

The resistance of erythrocytes to peroxidative lysis has been used for many years to evaluate the status of antioxidant defences (Gordon et al., 1955). Erythrocytes from individual fish which had been washed and diluted in 0.85% phosphate-buffered saline (PBS) were distributed into serial dilutions of H_2O_2 (hydrogen peroxide) in a 96-well plate. The resistance titre is the reciprocal of the highest dilution of H_2O_2 causing >50% lysis (evaluated visually) after overnight incubation at 4 °C.

Growth was expressed as the specific growth rate (SGR_W) and was determined from the following equation $SGR_W = 100\left(\text{Ln}W_f - \text{Ln}W_i\right)t$, where W_i and W_f are the initial and final weights (g) of the fish, respectively, and t is the duration (d) of the experiment (Ricker, 1979).

Statistical analyses

A rotatable 2^3 factorial-central composite design was used in this study (Box and Draper, 1987). The simultaneous effects of the three dietary nutrients (TOCAA, Se, and Fe) on oxidative stress were

determined by surface response methodology (Mead and Pike, 1975). A central composite design consists of a two-level full factorial design superimposed on a star design (axial points) (Box and Draper, 1987). The centres of the two designs coincide (centre point). In coded factor space, factorial points are located a distance ±1 from the centre, and axial points are located a distance ±α ($2^{k/4} = 2^{3/4} = 1.682$; k = number of dietary treatment factors) from the centre point and symmetrically positioned with respect to the factorial cube (Box et al., 1978).

Five different levels of each tested nutrient (TOC-AA, Se and Fe) were used to produce fifteen dietary combinations (diets) (Table 2). Twenty-four tanks received diets 1–8 (three replicates) used in the factorial portion of the design (Table 2). Diet 9, associated with the centre point of the design, was assigned to two tanks (two replicates), and diets 10–15, representing the axial points of the design, were assigned to six tanks (1 tank/diet). The centre point of the design is replicated so that the lack of fit of the response model can be estimated (Box et al., 1978). The axial points represent the dietary minimum and maximum for each experimental nutrient used in this study and were established as concentrations approaching dietary deficiency and toxicity in salmonids as determined from the published literature. The factorial and centre point levels of experimental nutrients used in diets were calculated by mathematical interpolation from the dietary minimum and maximum using the coded factors as described by Box and Draper (1987). For the diets used in the axial portion of the design, each nutrient was tested at its upper and lower level, while the other two nutrients were held at their centre point levels in the diet (Table 2).

Values from measures of oxidative stress in fish were averaged for each tank (experimental unit) for statistical analyses. To determine if tested nutrients affected oxidative stress, non-linear response surface models were generated for each measure of oxidative stress using multiple regression techniques and evaluated with the procedure outlined by Milliken and Johnson (1989). A brief description follows. First, the effect of each dietary nutrient was determined (F-test) after the adjustment of all other treatment factors. Dietary nutrients with an F-test, p-value ≤ 0.10 were included in the model for a particular measure of oxidative stress. Secondly, we determined the portion of the overall R^2 that was due to: (i) the linear terms in the model; (ii) the quadratic terms in the model after adjusting for the linear terms; and (iii) the cross-product terms in

the model after adjusting for both the linear and quadratic terms. All model terms with $R^2 \geq 0.200$ were considered to have a large effect and were included in the model. Surface response plots were constructed for each of the fitted models. Finally, lack-of-fit for each model was determined according to Khuri and Cornell (1996), where $F_{\text{Lack-of-fit}} = (\text{lack-of-fit mean square} \div \text{pure error mean square})$.

Results

Dietary TOCAA significantly affected (p ≤ 0.05) erythrocyte resistance to peroxidative lysis (ERPL) and liver lipid peroxidation (LPO) in December (16 weeks) and March (28 weeks) and liver protein carbonyl (PC) content in March. LPO and PC in liver and ERPL (December; lower values indicate increased resistance of erythrocytes to peroxidative lysis) decreased as TOCAA increased in the diet, and the lowest levels of these measures of oxidative stress were observed at the highest concentrations of TOCAA supplementation. The responses of liver LPO in December (Fig. 1; $R^2 = 0.22$. p = 0.02) and liver carbonyl in March (Fig. 2; $R^2 = 0.23$. p = 0.02) to TOCAA were quadratic, where the magnitude of the reduction increased with increasing level of TOCAA in diets. The effect of TOCAA on ERPL in December (Fig. 3; $R^2 = 0.36$. p = 0.005) and on liver LPO in March (Fig. 4; $R^2 = 0.27$. p = 0.03) was linear, with increasing levels of TOCAA producing a proportionate reduction in these measures of oxidative stress. The response model for ERPL in March was second order, containing linear ($R^2 = 0.34$. p = 0.01) and quadratic ($R^2 = 0.26$. p = 0.02) terms for TOCAA, and exhibited an optimum response (lowest values)
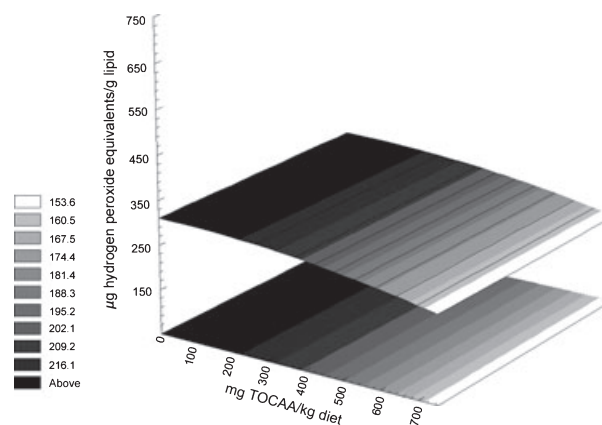

Fig. 1 Surface response plot of liver lipid peroxidation (μg H$_2$O$_2$ equivalents/g lipid) in December. Response model: $z = 223.0 - 1.4 \times 10^{-4}(\text{TOCAA})^2$. TOCAA, α-tocopherol + ascorbic acid.
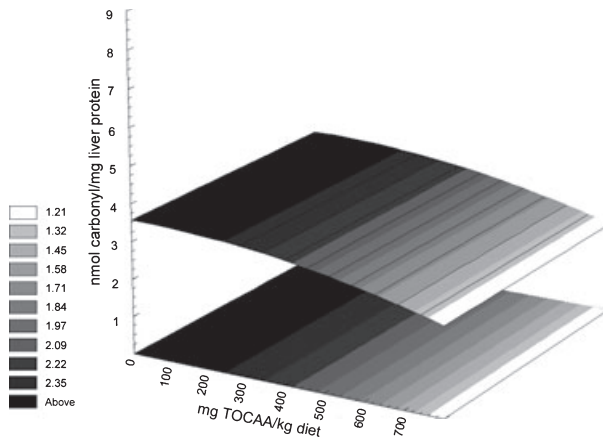
Fig. 2 Surface response plot of liver protein carbonyl content (nmol carbonyl/mg liver protein) in March. Response model: $z = 2.48 - 2.67 \times 10^{-6}(TOCAA)^2$. TOCAA, α-tocopherol + ascorbic acid.
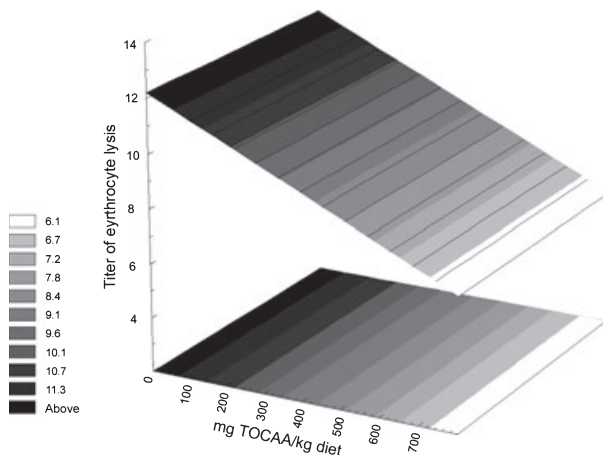

Fig. 4 Surface response plot of liver lipid peroxidation (μg H_2O_2 equivalents/g lipid) in March. Response model: $z = 281.2 - 1.7 \times 10^{-1}$ (TOCAA). TOCAA, α-tocopherol + ascorbic acid.

Fig. 3 Surface response plot of erythrocyte resistance to peroxidative lysis (titre of erythrocyte lysis) in December. Response model: $z = 11.8 - 7.9 \times 10^{-3}(TOCAA)$. Resistance titre is the reciprocal of the highest dilution of H_2O_2 causing >50% lysis. TOCAA, α-tocopherol + ascorbic acid.

Fig. 5 Surface response plot of erythrocyte resistance to peroxidative lysis (titre of erythrocyte lysis) in March. Response model: $z = 15.2 - 4.0 \times 10^{-2}(TOCAA) + 4.2 \times 10^{-5}(TOCAA)^2$. Resistance titre is the reciprocal of the highest dilution of H_2O_2 causing >50% lysis. TOCAA, α-tocopherol + ascorbic acid.

to 345–615 mg TOCAA/kg diet—ERPL decreased with increasing dietary TOCAA but began to increase when TOCAA in the diet exceeded 615 mg/kg (Fig. 5). The fitted response models are given in Table 3. Iron and selenium had no effect ($R^2 < 0.20$; $p > 0.10$) on measures of oxidative stress. No interactions were observed between tested nutrients.

Liver LPO in March was higher compared to December at the same dietary TOCAA levels (Figs 1 and 4). March ERPL was higher and, in contrast to December, more responsive to changes in dietary TOCAA at lower levels in the diet (approximate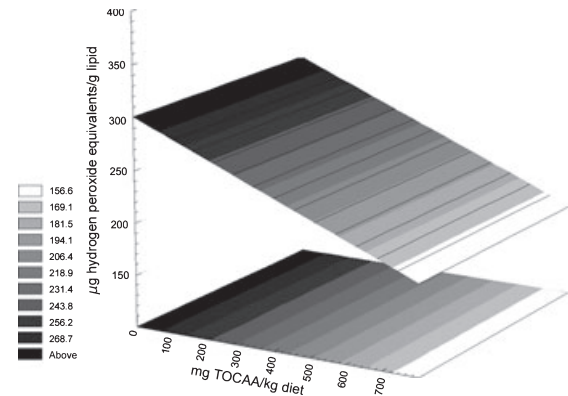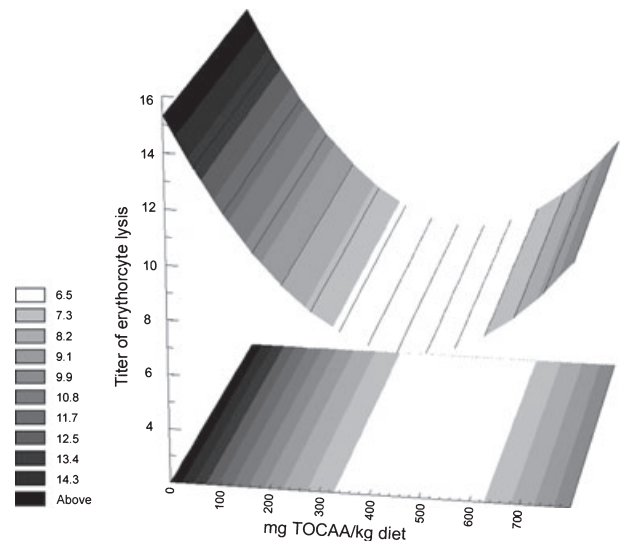ly 0–160 mg/kg diet), but the reverse was generally true at higher TOCAA levels (approximately 300–625 mg/kg diet) due to the quadratic component of the response (Figs 3 and 5).

The test for lack-of-fit was significant for liver LPO in December and liver carbonyl in March (Table 3). The lack-of-fit for these models may be a result of inadequate replication ($n = 2$) of the design centre point. Therefore, the fit of these models should be interpreted with caution. Diet did not affect growth rate or kidney LPO or PC content in Chinook salmon in this study ($p > 0.05$).

Table 3 Response surface models

| | Test for lack of fit | | |
Measured variable	F	P	Response model
Liver LPO December	5.46	0.01	$z = 223.0 - 1.4 \times 10^{-4}(TOCAA)^2$
ERPL December	0.81	0.64	$z = 11.8 - 7.9 \times 10^{-3}(TOCAA)$
Liver LPO March	0.92	0.55	$z = 281.2 - 1.7 \times 10^{-1}(TOCAA)$
Liver carbonyl March	2.96	0.02	$z = 2.48 - 2.67 \times 10^{-6}(TOCAA)^2$
ERPL March	0.80	0.65	$z = 15.2 - 4.0 \times 10^{-2}(TOCAA) +$ $4.2 \times 10^{-5}(TOCAA)^2$

TOCAA, α-tocopherol + ascorbic acid; LPO, lipid peroxidation; ERPL, erythrocyte resistance to peroxidative lysis.

Discussion

Dietary levels of α-tocopherol and ascorbic acid appeared to be adequate to counter oxidative stress in liver of parr in December, so that, in contrast to smolting fish sampled in March, less of these antioxidants was required to achieve comparable levels of protection against lipid peroxidation. The proportion of highly peroxidizable PUFAs in cellular membranes increases during smoltification (Henderson and Tocher, 1987). As tissue PUFA content increases, a higher level of vitamin E is required to counter peroxidative damage (Bell and Cowey, 1985). In Atlantic salmon smolts, the ratio of vitamin E to PUFA is probably critical in protection against lipid peroxidation, and so may mediate the vitamin E requirement (Hamre and Lie, 1995). The requirement for vitamin C undoubtedly increases as well, due to its role in returning vitamin E to the active state after interaction with free radicals (Hilton, 1989). Additionally, levels of T_3 and T_4 hormones and the activities of respiratory enzymes associated with aerobic energy production, which produce free radicals as a byproduct (Kehrer, 1993), are elevated in smolts compared to parr (Blake et al., 1984). These factors may explain why liver LPO was higher in March than in December and why less TOCAA was required to achieve a comparable level of protection against LPO in liver in December.

In contrast to December, erythrocytes in March were more susceptible to peroxidative lysis, more responsive to changes in dietary TOCAA when at lower dietary levels, and less responsive to higher levels. The erythrocyte fragility test has long been used as an indicator of vitamin E status (Woodall et al., 1964). Salmon erythrocytes are very high in PUFA: in Atlantic salmon, 54% of the fatty acids in erythrocyte membranes are n-3 PUFA (Thompson et al., 1995). It is likely that the parr-smolt transformation further increases the proportion of PUFA in salmon erythrocyte membranes, as it does in other tissues (Henderson and Tocher, 1987), though this has not been investigated to our knowledge. The response of March ERPL to dietary TOCAA illustrates the enhanced susceptibility of highly unsaturated cellular membranes to peroxidative damage when inadequate quantities of antioxidants are provided by the diet.

Approximately 345–615 mg TOCAA/kg diet provided the optimal response in the March ERPL assay (lowest values); when dietary ascorbic acid and/or α-tocopherol was higher than 615 mg/kg, the erythrocyte membranes of smolts were more susceptible to peroxidation. This was not seen in December, when n-3 PUFA levels of erythrocyte membranes were probably lower. Very high levels of vitamin C can lead to peroxidation of PUFA in some animals (Barja et al., 1996). In smelt (*Plecoglossus altivelis*), high dietary levels of α-tocopherol (1% of diet) also can promote lipid peroxidation of erythrocyte membranes (Kaewsrithong et al., 2001). The high proportion of n-3 PUFA in erythrocyte membranes probably makes them more sensitive and susceptible to oxidative damage when fish are fed either inadequate or elevated levels of some antioxidants, such as ascorbic acid.

Iron did not affect oxidative stress indices in this study. Dietary supplementation with excessive levels of Fe does not always promote oxidative damage to tissues (Desjardins et al., 1987; Lygren et al., 1999). In rainbow trout, iron supplied as ferrous sulphate produced toxic effects only when supplied at ≥1380 mg/kg in feed with low rancidity, but levels as low as 86 mg/kg produced iron overload when rancidity was high. Iron concentrations of 1300 mg/kg diet or lower had no effect on TBAR levels, a marker for lipid peroxidation, in fresh Atlantic salmon fillets (Torstensen et al., 2001). In the present study, the maximum level of dietary Fe was 750 mg/kg diet and probably not high enough to stimulate oxidative damage under the study conditions.

Interactions between ascorbic acid and iron supplemented in diets have been previously described in salmonids (Maage et al., 1990; Sandnes et al., 1990; Andersen et al., 1996). However, we did not observe an oxidative stress-related interaction between TOC-AA and Fe. Lygren et al. (1999) also did not find that supplementation of ascorbic acid and Fe at levels >1000 mg/kg diet, alone or in combination, caused an increase in oxidative stress in Atlantic salmon. Iron and ascorbic acid supplemented at lower levels, 400 mg/kg and 150 mg/kg diet, respectively, did not affect the antioxidant status of Atlantic salmon smolts (Andersen et al., 1998). The impact of dietary iron on oxidative stress in fish is complex and is probably dependent upon diet rancidity, the level and form of the supplemented Fe as well as levels of other antioxidants.

Dietary Se did not affect any of the indices of oxidative stress examined in this study. Poston et al. (1976) suggested that 0.03–0.04 mg Se/kg diet was sufficient for complete inhibition of *in vitro* lipid peroxidation in the presence of supplemental vitamin E, but that this level might be nevertheless suboptimal. In rainbow trout, the minimum required dietary level for Se has been estimated to be 0.07 mg/kg diet (Hilton et al., 1980) and 0.3 mg/kg diet (National Research Council (NRC), 1993). Felton et al. (1996) reported that juvenile salmon could tolerate dietary Se supplied as sodium selenite at levels as high as 13.6 mg/kg without toxic effects, and that 8.6 mg/kg Se produced total-body Se concentrations in hatchery coho salmon similar to concentrations in their wild counterparts. The findings of Felton et al. (1996) are contrary to previous reports for rainbow trout, where 13.0 mg/kg diet supplied as sodium selenite was toxic (Hilton et al., 1980), but are supported by the current study, which showed that the highest level of selenium tested, 9.0 mg/kg diet, produced no observable detrimental effects.

Previous studies have shown that the level of dietary Se has variable effects on oxidative stress indices, but effects appear to be small as long as Se is not deficient in the diet (Hilton et al., 1980; Felton et al., 1996). In the present study, α-tocopherol and Se did not appear to interact synergistically, contrary to some previous reports (Poston et al., 1976; Bell and Cowey, 1985; Bell et al., 1985). In rainbow trout (Hilton et al., 1980; Bell et al., 1985) and channel catfish (*Ictalurus punctatus* Rafinesque) (Gatlin et al., 1986), no specific deficiency signs of Se were observed as long as dietary vitamin E was adequate. The lowest level of α-tocopherol used in this study may have been sufficient to offset any effects of low dietary Se. In addition, there are probably age and species specific differences in the interaction of vitamin E and Se (Hilton, 1989), which may in part account for the absence of an interaction.

Conclusions

Of the nutrients tested, only TOCAA had an effect on oxidative stress. Generally, measures of oxidative damage to tissues decreased with increasing TOCAA in diet, but we observed a TOCAA-stimulated increase in ERPL values (higher values indicate decreased resistance to lysis) in March at the highest levels of TOCAA (750 mg/kg) in diet. The data suggest that oxidative stress progressively decreases as TOCAA increases in the diet up to an optimal range, but that higher TOCAA concentrations can prove detrimental to fish. Although the minimum requirements in salmon for vitamin E are 30–50 mg/kg diet and for ascorbic acid 50 mg/kg, Halver (2002) suggests a dietary ascorbic acid level as high as 200 mg/kg to compensate for losses during feed processing and storage and to ensure adequate tissue reserves. In our study, erythrocytes appeared to be more sensitive to variation in dietary levels of TOCAA than liver and kidney tissues. Using the March ERPL assay results as a baseline, α-tocopherol and ascorbic acid levels of approximately 350–600 mg/kg diet would provide adequate protection against lipid peroxidation.

Higher levels of TOCAA in diet were required in March than in December to achieve comparable levels of protection against oxidative damage, likely due to physiological changes associated with the parr-smolt transformation. Dietary levels of antioxidants well above minimum physiological requirements would likely benefit fish exposed to periodic oxidative stress, such as during disease infection or episodes of poor water quality. The level of peroxidative damage required to compromise physiological function or biological fitness has been little studied in fish and is poorly understood for mammals, further complicating recommendations for optimal dietary supplementation levels. Additional examination of the effects of dietary antioxidants and their interactions on oxidative stress is needed in relation to important measures of biological fitness, such as immune function and stress tolerance, to further elucidate appropriate levels of antioxidants in the diets of cultured salmonids.

Acknowledgements

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